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Therapeutic evaluation of *Murraya koenigii* in bovine subclinical mastitis

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Abstract
The present study was aimed to evaluate *Murraya koenigii* leaves for *in vitro* antibacterial activity of its hydro-alcoholic extract against common bovine mastitogens, and thereafter, *in vivo* therapeutic efficacy in subclinical mastitis affected dairy cows. The final yield of herbal extract (g/ 100g of herb) from *M. koenigii* leaves was 7.8%. The antibacterial activity of the hydro-alcoholic extract in terms of the average zone of inhibition shown by *M. koenigii* against *S. aureus* and *E. coli* were 14.13 mm and 16.00 mm respectively and the respective minimum inhibitory concentration was 31.25 mg/ml and 125 mg/ml. *In vivo* therapy of the herb against specific subclinical mastitis of cows revealed significant decline in total bacterial count, California Mastitis Test score, Somatic Cell Count, electrical conductivity, pH and lymphocytes while there was significant increase in milk neutrophils, and effect on the elimination of intra mammary infections (50.00%) against control (25.00%) on day 28 of treatment was found non-significant (χ^2 at 01df, N=64 = 1.641, p > 0.5). Therapy with *M. koenigii* showed significant effect on minimizing the udder inflammation and improvement of milk quality; however, nonsignificant effect on elimination of intra mammary infections warrants more research while using this herb or its pure active constituents in bovine sub-clinical mastitis.

Keywords: *Murraya koenigii*, bovine subclinical mastitis, *in vitro* antibacterial activity, intramammary infections

Introduction
Maharashtra state stands 7th in livestock population and 5th in cattle population. During the year 2019, the total cattle population in the state was 13.9 million (20th Livestock Census). The milk production in the state during 2018-2019 was recorded to be 2354.32 lakh MT with per capita availability of 394 grams/day. Mastitis is a complex disease found in clinical and sub-clinical types (Quinn *et al.*, 1999) [36]. Clinical mastitis is characterized by visible signs of udder inflammation and gross abnormality in milk, and is usually referred to as an individual health problem, whereas, subclinical mastitis is absent of any apparent manifestation of inflammation and is characterized by no noticeable signs in the udder or milk, however milk production is decreased and the composition of milk is changed (Guidry, 2007) [16]. Subclinical mastitis is 3-40 times more prevalent than clinical mastitis, and therefore, is economically more destructive and clinically elusive due to its camouflaged clinical manifestations (Daimi *et al.*, 2006; Tesfaye *et al.*, 2010; Bachaya *et al.*, 2011; Leitner *et al.*, 2012; Shaikh *et al.*, 2018) [7, 46, 4, 24, 43]. Current mastitis therapy focuses primarily on the administration of antibiotics to the mastitic animal. The greatest risk, however, is the emergence of antibiotic resistance (Van Hoek *et al.*, 2011) [47], and presence of antibiotic residues that pose a threat to public health. For this reason, it become imperative to use non-antibiotic approaches based on improving the natural defence mechanism of the animal by using non-specific immune-modulators such as plant products. Due to their lower toxicity, lower side effects and organic nature, the herbal medicine has attained significance. Furthermore, herbal therapy usually does not influence or change the milk quality and does not require milk withdrawal period as is mandatory for antibiotic use.

Murraya koenigii (Curry) leaf is an important leafy vegetable that belongs to the Rutaceae family, which is native to India and the Southeast Asian Region. In addition, with number of important health benefits the curry leaves are used as natural flavouring agent. It contains several medicinal properties such as anti-diabetic, anti-oxidant, anti-microbial, anti-fungal, anti-inflammatory, anti-carcinogenic, hepato-protective, cardio-protective, anti-ulcer activity, anti-diarrheal activity and phagocytic activity.

The chemical composition of the fresh leaves of *M. koenigii* consists of volatile oils, and carbazole alkaloids and triterpene have been isolated from its roots and stem bark (Bhandari, 2012 and Disegha, *et al*., 2014) [6, 10].

Materials and Methods

In vitro antibacterial activity of leaf extract

The antibacterial activity of the hydro-alcoholic herbal extract of *M. koenigii* leaves was determined against common mastitis pathogens isolated in this study. The leaves of the herb were collected from the botanical garden that were initially air dried and later on in the incubator at 40 ºC. to remove excess moisture. Preparation of the leaf extract and *In vitro* antibacterial activity of leaf extract was performed as per the procedure (Shafi *et al* 2020) [39]. The antibacterial activity of the hydro-alcoholic herbal extract was determined against common mastitis pathogen on the basis of disappearance of turbidity/ minimal inhibitory concentration (MIC) in the tube dilution method. For it, serial dilutions of the extracts will be prepared from 500 mg/ml dilution with the help of tween-20. The lowest concentration of extract preventing the growth on the plate will be taken as MIC for that extract.

In vivo therapeutic potential of herbs

For *In vivo* studies cows found positive for specific subclinical mastitis in at least one of the quarters were selected and randomly assigned into treatment and control groups taking into consideration specific physiological data such as calving, lactation stage and milk yield etc. Treatment group (n= 08) received curry leaves powder @ 60 mg / Kg BW orally bid for seven days and the dose of herb was calculated on the basis of following facts. Ghosh *et al* (2013) [14] reported the LD50 of pure aqueous extract of *M. koenigii* as 150 mg/kg body weight. For safety reason $1/10^{th}$ of its LD50 (15 mg/kg) was taken and Wynn and Fougere (2007) has recommended crude powder four times the dose of its herbs pure extract i.e. @ 60 mg/kg body weight (24 g total dose). Animals in the control group (n=08) were given placebo (wheat bran).

Sampling and parameters studied

To assess the quarter health status, milk quality and immune status of udder, quarter foremilk (QFM=10 mL) and Cow composite milk (CCM=40 mL) samples were collected pre-treatment (d 0), and post-treatment d 7, d14 and d 28 and were analyzed for various parameters. Isolation and identification of bacteria from QFM samples was performed as per the standard microbial procedures of National Mastitis Council (1990) [29]. Modified California Mastitis Test (MCMT) was performed as per the method described by Pandit and Mehta (1969) [34], Milk pH was determined by an electronically operated single electrode Pen type digital pH meter, the electrical conductivity of milk was measured by an EZ-1 pen type electrical conductivity meter, Somatic Cell Count (SCC) was determined as described by Schalm *et al.* (1971) [38], biochemical composition parameters of the milk i.e. fat, SNF, protein and lactose were analyzed by Milk analyzer, Total bacterial count of milk samples were assessed as per standard protocol, Differential leukocyte count in milk was assessed as per Dulin *et al* (1988).

Statistical analysis

The data were processed via the statistical package for social science (SPSS version 16.0 for windows) using ANOVA, followed by Duncan's multiple range test and the data on elimination of intramammary infections was analysed using Chi square test. Significance level was set at $P \leq 0.05$.

Results and Discussion

The *in vitro* antibacterial activity of hydro-alcoholic extracts of *M. koenigii* leaves was studied against the common mastitis pathogens viz., *S. aureus*, *E. coli*, *Pseudomonas* spp. and *Corynebacterium* spp. The final yield of herbal extract (g/ 100 g of herb powder) obtained from *M. koenigii* leaves was 7.8%. The MIC and zones of inhibition produced by *M. koenigii*, and their antibacterial activity in terms of percent inhibition in comparison to enrofloxacin for different bacteria are given in Table 1.

Table 1: Zones of inhibition and MIC of hydro-alcoholic extract of *M. koenigii*

Pathogen	Zones of Inhibition (mm)		% inhibition	MIC (mg/ml) (*M. koenigii*)
	M. koenigii	Enro.		
Staphylococcus aureus	14.13	20.75	68.10	31.25
Corynebacterium spp.	15.00	24.88	60.30	31.25
E. coli	16.00	20.75	77.11	125
Pseudomonas spp.	14.00	24.38	57.44	125

Various researchers have evaluated the antibacterial activity of *M. Koenigii* leaves and have found promising activity against various microorganisms. Mathur *et al.* (2010) [25] studied antimicrobial activity of hexane and methanolic leaf extracts of *M. Koenigii* against aerobic bacteria associated with bovine mastitis and observed methanolic extract inhibiting growth of *S. aureus, S. epidermidis, S. uberis, P. aeruginosa, E.coli, Corynebacterium gravis* and *Bacillus cereus*, while as, hexane extract could inhibit growth of all the selected microorganisms except *S. epidermidis, S. uberis and Bacillus cereus*. Antibacterial activity of various types of extracts of different parts of *M. Koenigii* against various microorganisms have been evaluated showing promising results by various researchers such as; Ningappa *et al.* (2010) [31], Gupta *et al.* (2011) [17], Handral *et al.* (2012) [19], Saini and Tyagi (2015) [37], Akula *et al.* (2016) [2], Harbi *et al.* (2016) [3] and Yin *et al.* (2018) [49]. Curry Leaf (*Murraya koenigii*) is an important leafy vegetable and natural flavoring agents with a number of important health benefits possessing several medicinal properties such as anti-diabetic, antioxidant, antimicrobial, anti-fungal, anti-inflammatory, anti-carcinogenic and hepato-protective properties. The various notable pharmacological activities of the plant include activity on heart, anti-diabetic and cholesterol reducing property, antimicrobial activity, antiulcer activity, antioxidative property, cytotoxic activity, antidiarrheal activity, phagocytic activity. The chemical composition of the fresh leaves of *M. koenigii* consists of volatile oil. Carbazole alkaloids and triterpene have been isolated from stem bark and roots of *M. koenigii*. Gahlawat *et al.* (2014) [12] reported diverse role of *M. koenigii* in traditional medicine and therapeutic potential of leaves and roots, due to presence of various active constituents such as carbazole alkaloids phytocompounds like koenimbine, koenine, mahanimbine, murrayazolidine,

murrayazoline, murrayacine, girinimbine, mukoeic acid, possessing antioxidant, antimicrobial, anthelmintic, analgesic, anti-inflammatory, antidiarrheal, hepatoprotective and antitumor properties. Sharma *et al*. (2011) [44] reported that essential oil from *M. koenigii* leaves showed antibacterial effect against *B. subtilis, S. aureus, C. pyogenes, P. vulgaris and Pasteurella multicida I* and fractionation of acetone extract of the leaves of *M. koenigii* yeild three bioactive carbazole alkaloids, mahanimbine, murrayanol and mahanine, which have got mosquitocidal, antimicrobial and topisomerase I and II inhibition activities. The study listed various activities of *M. koenigii* such as antioxidant, antidiabetic, antibacterial, antihypertensive, cytotoxic and effective in the treatment of bronchial respiratory difficulties. Ganesan *et al.* (2013) [13] reviewed the bioactive compounds in *M. koenigii* and *Coriandrum sativum* and reported presence of many bioactive compounds like polyphenols, alkaloids and flavonoids having multiple bioactive functions like antioxidant, anticancer, antimicrobial antidiabetic and hepatoprotective. Mathur *et al.* (2010) [25] reported MIC of the methanolic leaf extracts of *M. Koenigii* as 8.25 mg/ml for *E. coli*, *S. epidermidis*, and *B. cereus*; 12.5 mg/ml for *S. aureus* and *S. uberis*, and 30 mg/ml for *P. aeruginosa* and *C. gravis*. Kumari *et al.* (2017) [23] reported MIC of 7.81 mg/mL and 15.62 mg/mL of curry leaves extract against *S. aureus* and *Micrococcus luteus*, respectively. Gupta *et al.* (2011) [17] evaluated various parts of *Murraya Koenigii* for antimicrobial properties and observed ethanolic extract of leaves was active against *S. aureus* and *B. subtilis* at a concentration of 6.25-12.5 mg/ml, with a median inhibition zone diameter of 20 mm. Whereas extract from the bark was active against *Aspergillus Niger* and *Candida albicans* at a concentration of 6.25-12.5 mg/ml with a maximum inhibition zone of 16-18 mm diameter. Handral *et al.* (2012) [19] studied *in vitro* antimicrobial activities of aqueous, chloroform, methanol and petroleum ether extracts from *Murraya koenigii* against pathogenic bacteria and observed that methanol extract having activity against *K. pneumoniae* and *S. typhi* while as, other three extracts showed activity mainly against *P. aeuriginosa*, *E. coli*, and *S. typhi*. The corresponding MIC and MBC values of the water, ethanol, chloroform, and petroleum ether extracts were 12.5 to 100.0 mg/ml and 50.0 to 100.0 mg/ml, respectively.

Elimination of intramammary infections at d28 post-treatment with *M. Koenigii* therapy (12/20; 50.00%) was found statistically non-significant (χ^2 = 1.641; 01 df; p > 0.05) (Table 2). Several researchers have evaluated different herbs for elimination of intra mammary infection and have reported beneficial effects of herbal therapy against bovine mastitis. Giacinti *et al.* (2008) [15] evaluated the effects of feeding of herbal extracts (5 gr. of standardised fluid extract of *Spirea ulmaria* L. and 6 gr. of standardised extract of *Astragalus*) for the control of bovine subclinical mastitis during lactation and observed reduction in infected quarters from 32.7 to16.7% in treated group vs. 35.4 to 30.2% in control group (P<0.05) from day 0 to day 56.

Table 2: Effect of herbal therapy on level of intramammary infections (IMI)

Organism	Intramammary infections (IMI) Vs. Treatment Group			
	Control (G1)		*M. Koenigii* (G2)	
	Present at 0d	Eliminated at 28d	Present at 0d	Eliminated at 28d
Staphylococci	13	2	13	7
Corynebacteria	3	1	4	2
Gram negative	4	2	3	1
Overall	20	5 (25.00)	20	10 (50.00)*

Figures in parentheses indicate percentage

Significant differences existed in elimination of IMI between treatment and control groups

* χ^2 = 1.641; 01df; p > 0.05

Shafi *et al.* (2016) [41] evaluated the immunotherapeutic potential of *O. sanctum* and observed elimination of intramammary infections in *O. sanctum* treatment (9/13; 69.23%) vs. control (4/15; 26.67%) were statistically significant (P ≤ 0.5) with χ^2 values being 5.07. Also Shafi *et al*. (2020) [39] while evaluating *W. somnifera* root powder observed that therapy could eliminate 64.28% of intramammary infections (χ2 = 4.14; 01 df; P<0.05). Patel and Gupta (2020) [35] evaluated a non-antibiotic therapy (Magic-3) for specific subclinical mastitis in crossbred cows and the therapy could eliminate 70.73% and 80.49% infection on d 15 and d 30, respectively.

Therapy with *M. koenigii* (G3) showed non-significant decline in TBC on day 7 (29.00 ± 1.51) in comparison to day 0 (34.50 ± 2.73) but significant decline in TBC on day 14 (22.63 ± 3.73) and day 28 (10.88 ± 2.89) (Table 3). There is a positive correlation between total bacterial count and SCC in milk from subclinical mastitis affected cows (Silva *et al.* 2008) [45], and as the infection of udder increases, SCC starts increasing proportionately. Likewise, elimination of intra mammary infections from the udder results in the reduction of SCC and TBC. Although there was a significant decline in the mean neutrophil percentage and significant increase in the mean lymphocyte percentage on day 28 of the trial in case of the control group overall the mean neutrophil percentage and lymphocyte percentage indicated the presence of the infection in the control group. Herbal therapy with *M. koenigii* resulted in the significant decrease in the mean neutrophil percentage and significant increase in the mean lymphocyte percentage from day 14 onwards that was also observed on day 28 (Table 3). Bovine mastitis results in the increase in the SCC that leads to simultaneous increase of neutrophils and it has been reported that neutrophils are the predominant in mastitis milk followed by lymphocytes, epithelial cells and monocytes (Dhakal and Kapur 1992) [9]. De *et al.* (2009) [8] studied the immunotherapeutic potential of hydro- methanolic extract of *A. indica* (intramammary infusion) by observing parameters such as, somatic cell count (SCC), total bacterial count (TBC), and milk differential leukocyte count, and observed significant reduction (P<0.05) in SCC neutrophil count however, and milk lymphocyte percent was significantly increased (P<0.05). Leukocytes are crucial for host innate immune response in dealing with the intra mammary infections, and early in the infection neutrophils are recruited due to their ability to phagocytose and kill bacteria. During mastitis polymorphonuclear leukocytes predominate in the mammary secretions for phagocytosis and intracellular killing of the invading pathogens by respiratory burst and activation

of lymphocytes by herbal therapy is indication of immunomodulation. Various researchers have reported the positive impact of herbal therapy in enhancing the phagocytosis ability of polymorphonuclear leukocytes and thereby leading to immunomodulation, a mechanism important for elimination of intra-mammary infections and subsiding of underlying udder pathogenesis and inflammation (Gupta and Pachauri 2001, Acharya *et al.* 2002, Mukherjee *et al*. 2005, Shafi *et al.* 2016, Shafi *et al.* 2018, Shafi *et al.* 2020) [18, 1, 27, 41, 40, 39].

The herb, *M. koenigii* have proven immunotherapeutic potential that results in the elimination of infection and undergoing inflammation resulting in decrease in the TBC and SCC (De *et al.* 2009) [8]. Therapy with *M. koenigii* showed non-significant decline in CMT point score on day 7 (1.50 ± 0.19) but significant decline was observed on day 14 (0.88 ± 0.23) and day 28 (0.38 ± 0.18) and similar trend for SCC was observed (Table 3). In case with *M. koenigii* therapies EC and pH of the milk showed significant improvement (Table 3) at the end of the therapy and therefor indicate positive impact of the therapy in terms of reducing the udder inflammation and improvement of the milk quality. Similarly various researchers have reported beneficial role of herbal therapy in terms of reducing udder inflammation, thereby improving milk parameters such as SCC, CMT, EC and pH (Gupta and Pachauri 2001, Nayak 2003, Mukherjee *et al*. 2005, Mir 2009, Bansal *et al.* 2013, Mukherjee *et al.* 2014, Shafi *et al.* 2016, Shafi *et al*. 2018, Shaikh *et.al*., 2019; Shafi *et al*. 2020, Patel and Gupta 2020) [18, 42, 5, 26, 27, 28, 41, 40, 39, 35].

Table 3: Effect of *M. koenigii* therapy on TBC, DLC and inflammatory markers of the udder

Parameters	Group	Days after initiation of treatment (AT)			
		Day 0	Day 7	Day 14	Day 28
TBC (10^4 cfu/ml)	G1	31.25 ± 2.32 ^{1a}	23.63 ± 1.08 ^{1b}	26.00 ± 2.89 ^{1ab}	26.00 ± 1.92 ^{1ab}
	G3	34.50 ± 2.73 ^{1a}	29.00 ± 1.51 ^{2ab}	22.63 ± 3.73 ^{1b}	10.88 ± 2.89 ^{2c}
Neutrophil (%)	G1	54.88 ± 2.27 ^{1a}	51.50 ± 0.98 ^{1ab}	52.25 ± 2.02 ^{1ab}	49.13 ± 1.46 ^{1b}
	G2	53.63 ± 3.08 ^{1a}	45.13 ± 2.22 ^{2a}	37.13 ± 2.35 ^{2c}	29.63 ± 1.77 ^{2d}
Lymphocyte (%)	G1	16.63 ± 0.94 ^{1a}	18.13 ± 0.88 ^{1ab}	19.75 ± 0.84 ^{1b}	19.00 ± 0.60 ^{1ab}
	G2	20.75 ± 0.73 ^{2a}	19.50 ± 0.63 ^{1a}	17.00 ± 0.76 ^{2b}	15.88 ± 0.74 ^{2b}
CMT point score	G1	2.13 ± 0.23 ^{1a}	1.50 ± 0.19 ^{1a}	1.75 ± 0.25 ^{1a}	1.50± 0.19 ^{1a}
	G2	1.88± 0.23 ^{1a}	1.50± 0.19 ^{1a}	0.88± 0.23 ^{2b}	0.38± 0.18 ^{2b}
SCC ($\times 10^3$ /ml)	G1	742.25± 59.65 ^{1a}	573.13± 46.37 ^{1ab}	623.63± 66.26 ^{1ab}	553.50± 48.09 ^{1b}
	G2	737.25± 61.99 ^{1a}	579.88± 41.48 ^{1a}	387.75± 63.90 ^{2b}	261.63± 50.75 ^{2b}
EC (mS/cm)	G1	5.47 ± 0.16 ^{1a}	5.49 ± 0.05 ^{1a}	5.41 ± 0.12 ^{1a}	5.17 ± 0.06 ^{1a}
	G2	5.48 ± 0.09 ^{1a}	5.29 ± 0.07 ^{2a}	5.00 ± 0.13 ^{2b}	4.62 ± 0.10 ^{2c}
pH	G1	6.87 ± 0.03 ^{1a}	6.72 ± 0.03 ^{1b}	6.74 ± 0.02 ^{1b}	6.74 ± 0.02 ^{1b}
	G2	6.86 ± 0.03 ^{1a}	6.77 ± 0.02 ^{1b}	6.69 ± 0.02 ^{1c}	6.65 ± 0.02 ^{2c}

Superscripts in each row (a, b, c) and each column (1, 2) differ significantly (P<0.05).

4.2.3 Effect on biochemical composition of milk

Therapy with *M. koenigii* showed significant increase in milk protein, fat and lactose on day 28, but there was no improvement in terms of SNF (Table 4). Subclinical mastitis in cows has been observed to deteriorate the milk composition in terms of reducing the percentage of fat, SNF, protein and lactose. Bansal *et al.* (2013) [5] studied the effect of Mastilep gel, an herbal preparation in mastitis affected cows and observed an significant increase in fat (3.56± 0.78 vs. 3.12 ± 0.85, p < 0.01) and lactose (5.07 ± 0.46 vs. 4.90 ± 0.37, p < 0.05).

Table 4: Effect of *M. koenigii* therapy on milk composition

Parameters	Group	Days after initiation of treatment (AT)			
		Day 0	Day 7	Day 14	Day 28
Protein (%)	G1	2.86 ± 0.08 ^{1a}	2.80 ± 0.15 ^{1a}	2.98 ± 0.12 ^{1a}	3.46 ± 0.18 ^{1b}
	G2	2.83 ± 0.07 ^{1a}	2.80 ± 0.05 ^{1a}	2.88 ± 0.06 ^{1a}	3.33 ± 0.06 ^{1b}
Fat (%)	G1	2.85 ± 0.35 ^{1a}	3.14 ± 0.28 ^{1a}	3.07 ± 0.38 ^{1a}	3.16 ± 0.33 ^{1a}
	G2	2.81 ± 0.20 ^{1a}	2.73 ± 0.16 ^{1a}	3.04 ± 0.25 ^{1a}	3.61 ± 0.13^{1b}
SNF (%)	G1	7.95 ± 0.20 ^{1a}	7.77 ± 0.27 ^{1a}	7.98 ± 0.20 ^{1a}	8.06 ± 0.13 ^{1a}
	G2	7.75 ± 0.16 ^{1a}	7.95 ± 0.15 ^{1a}	7.79 ± 0.18 ^{1a}	7.79 ± 0.08 ^{1a}
Lactose (%)	G1	4.30 ± 0.11 ^{1a}	4.28 ± 0.06 ^{1a}	4.43 ± 0.08 ^{1a}	4.51 ± 0.07 ^{1a}
	G2	4.21 ± 0.09 ^{1a}	4.21 ± 0.09 ^{1a}	4.46 ± 0.05 ^{1b}	4.60 ± 0.07 ^{1b}

Superscripts in each row (a, b, c) and each column (1, 2) differ significantly (P<0.05).

Nurdin *et al.* (2011) [33] studied the effects of herbs *(Black Cumin, Curcuma zeodharia, Curcuma mangga, and Curcuma aeruginosa)* supplementation on milk yield and milk quality and observed significant increase (P<0.01) in milk yield, milk protein, milk lactose, however, did not affect milk fat significantly. Khanal *et al.* (2017) studied the effect of three different treatments namely, T1 = T2 + powder of Neem leaves (i.e. normal feeding + 500g UMMB + neem powder), T2 =T1 + 500 g UMMB/day (normal feeding + UMMB), T3 = normal feeding (control), and observed significant increase (P < 0.05) in the milk yield (kg) and milk lactose. Promising results of herbal therapy in terms of improving the milk biochemical composition and overall improvement of the milk quality have been observed by various researchers as well (Jaguezeski *et al.* 2018, Nurdin 2018, Shafi *et al*. 2016, Shafi *et al*. 2018, Harjanti *et al.* 2019, Shafi *et al*. 2020) [21, 32, 41, 40, 39].

Conclusions

Therapy with *M. koenigii* showed significant effect on minimizing the udder inflammation and improvement of milk quality; however, nonsignificant effect on elimination of intra

mammary infections warrants more research while using this herb or its pure active constituents in bovine sub-clinical mastitis.

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